A NATURAL HYBRID BETWEEN *LIGULARIA PARADOXA* AND *L. DUCIFORMIS* (ASTERACEAE, SENECIONEAE) FROM YUNNAN, CHINA[1]

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Abstract

A putative natural hybrid in *Ligularia* Cass., which is morphologically intermediate between *L. paradoxa* Hand.-Mazz. and *L. duciformis* (C. Winkl.) Hand.-Mazz., was found on Mt. Maoniu in northwestern Yunnan, China. We employed analyses of morphology, seed vigor, somatic chromosome numbers, meiotic behavior of chromosomes, inter-simple sequence repeat (ISSR) markers, and *trnL-F* sequencing to test the hypothesis that the unidentified taxon is a natural interspecific hybrid between *L. paradoxa* and *L. duciformis*. Examination of morphological characters demonstrated the intermediacy of the putative natural hybrid between *L. paradoxa* and *L. duciformis*. Seed germination of the putative natural hybrid was absent, and no embryo was found in seeds of the putative natural hybrid. The meiotic behavior of the putative natural hybrid was abnormal. Among 10 ISSR primers that we tested in all three taxa, four primers produced different ISSR markers between the putative parents. The putative natural hybrid shared all ISSR markers of the putative parents. However, no taxon-specific band existed in the samples of the putative natural hybrid. The data from ISSR markers are concordant with morphology in implicating *L. paradoxa* and *L. duciformis* as parents of the putative natural hybrid. *Ligularia paradoxa* was determined as the female parent of this natural hybrid by nucleotide sequence from the chloroplast *trnL-F* region. Therefore, this taxon is confirmed to be a natural hybrid between *L. paradoxa* and *L. duciformis* and is described herein.

Key words: Asteraceae, IUCN Red List, *Ligularia*, natural hybrid, Senecioneae.

Hybridization is common in many groups of flowering plants (Stebbins, 1959; Grant, 1981; Ellstrand et al., 1996) and is increasingly recognized as an important source of evolutionary novelty (Arnold, 1997; Rieseberg, 1997). Hybrids can arise in any situation where genetic divergence is not closely accompanied by development of a reproductive barrier, thus creating the potential for gene flow between entities that are taxonomically recognizable and morphologically distinct. The consequences of hybridization may include introgression affecting one or both taxa, formation of hybrid (especially alloploid) species, and development of reticulate patterns of evolution within a group (Arnold, 1997).

Although hybridization in combination with polyploidy has long been accepted as a mechanism of speciation in plants (Stebbins, 1971; Levin, 1983), the increasing use of molecular approaches has facilitated understanding of this process and revealed many new examples (Barton, 1989; Wendel, 1989; Soltis et al., 1991; Harris & Ingram, 1992; Soltis & Soltis, 1993; Brochmann et al., 1996; Sun, 1996; Sang et al., 1997; Padgett et al., 1998; Wolfe et al., 1998; Aparicio et al., 2000; Barton et al., 2001; Choi et al., 2001; Allen, 2002). Molecular data can yield many insights into the nature and parentage of hybrid species and, as further cases are investigated, into the circumstances that favor allopolyploid speciation. They are most commonly observed in outcrossing, perennial taxa, typically those with some mode of vegetative propagation.

The genus *Ligularia* Cass. (Asteraceae, Senecioneae) consists of about 130 species mainly distributed in eastern Asia, 112 species of which are known in China (Liu et al., 1994). Sixty-seven species were recognized in the Hengduan Mountains in southwestern China; of them, 61 species are endemic to this area (Liu, 1989). The Hengduan Mountains have been

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considered a main center of evolution and diversification of *Ligularia* (Liu et al., 1994). *Ligularia* is a typical alpine genus and a key taxon to studies on the origin and development of the Sino-Himalayan alpine floristic elements of flora. Although *Ligularia* has been taxonomically studied by Handel-Mazzetti (1938), Hu (1967), and Liu (1985, 1989), no molecular phylogenetic studies have been reported yet. The authors are doing a comprehensive study on the phylogeny of *Ligularia* using various methods to investigate its morphology, cytology, and molecular biology.

During field collection trips to the Hengduan Mountains in Yunnan, we found a mixed population of three taxa, namely *Ligularia paradoxa* Hand.-Mazz., *L. duciformis* (C. Winkl.) Hand.-Mazz., and a putative natural hybrid, occurring on Mt. Maoniu. This mixed population grows along a stream in the forest at an altitude of 4200 m. The putative nothospecies was considered to be a natural hybrid between *L. paradoxa* and *L. duciformis* based on its intermediate morphological appearance and the partial overlap of the flowering period of two putative parents. Given this anthesal overlap, cross-pollination between two putative parents could occur naturally under sympatric conditions.

The aim of the present study is to clarify the taxonomic status of the putative natural hybrid of *Ligularia duciformis* and *L. paradoxa* found on Mt. Maoniu, and to determine systematic relationships among these three taxa using various taxonomic approaches, including comparison of external morphology, seed vigor, examination of somatic chromosome number and meiotic behavior, inter-simple sequence repeat (ISSR) markers, and *trnL-F* sequencing.

MATERIALS AND METHODS

Morphological observation was carried out in the field during the 2001 and 2002 flowering seasons. Leaf form and pappus color were selected among morphological characters as diagnostic characters. For studies on seed vigor, achenes of the three taxa were collected in October 2001. Embryos of all three taxa were observed under a light microscope. Achene weights were measured based on 1000 achenes, and the germination rates of achenes, sown in soil, were counted.

Somatic chromosome numbers were examined by the conventional aceto-orcein squash method using root tips. Root tips were collected from five living individuals per taxon in the field and then were pretreated with 0.1% colchicine solution for 3 hr. After fixation with a 3:1 mixture of 95% ethanol and glacial acetic acid at 4°C for 15 min., root tips were macerated in a 1:1 mixture of 45% acetic acid and 1 M hydrochloric acid at 60°C for 3 min., and then stained with 1% aceto-orcein. To study meiosis, young calathides of the three taxa were fixed in Carnoy's solution (3:1 ethanol:acetic acid) in the field, then transferred to 70% ethanol and refrigerated. Squashes were made in a drop of aceto-orcein.

For study of both ISSR markers and the sequences of the chloroplast *trnL-F* region, the young leaves of 56 individuals (20 of *Ligularia duciformis*, 20 of *L. paradoxa*, and 16 of the putative natural hybrid) were collected and dried in silica gel in the field. Total genomic DNA was extracted from the silica gel–dried leaf tissue using CTAB methods (Doyle & Doyle, 1987). All 90 ISSR primers were used for screening all individuals, and 10 primers (University of British Columbia, Canada [UBC] primers set #9: 807, 808, 809, 815, 820, 823, 827, 828, 834, 848) were selected for amplifying bands. The polymerase chain reaction (PCR) included the following cycles: preheat, 94°C, 5 min.; 45 cycles, denaturation, 94°C, 1 min.; annealing, 52°C, 1 min.; extension, 72°C, 1.5 min.; final extension, 72°C, 7 min. The amplified bands from three taxa were scored as (1) common to both putative parents, (2) shared with one of the putative parents, or (3) unique to the putative natural hybrid. The chloroplast *trnL-F* region was amplified using the universal primers *trnC* and *trnF* (Taberlet et al., 1991). Amplification conditions were as follows: 1 cycle, 94°C, 4 min.; 28 cycles, 94°C, 45 sec.; 58°C, 45 sec.; 72°C, 2 min., followed by 1 cycle, 72°C, 8 min. The PCR products were purified by electrophoresis through a 1.2% agarose gel followed by use of an E.Z.N.A. Gel Extraction Kit (Omega, Guangzhou, China). All accessions were subjected to chloroplast *trnL-F* sequencing, with amplification primers in an ABI 3700 DNA automated sequencer with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, U.S.A.). Boundaries were determined by comparison with *Nicotiana paniculata* L. (AY098701). All sequences have been deposited in GenBank (accession numbers DQ104429–DQ104431). The sequences were aligned and compared in SeqMan (DNASTAR, Beijing, China).

RESULTS

MORPHOLOGICAL ANALYSIS

Ligularia duciformis and *L. paradoxa* belong to series *Retusae* S. W. Liu (Liu, 1989). The main morphological differences between the two species are leaf form and pappus color (Table 1, Fig. 1). The

Table 1. Morphological comparison of *Ligularia paradoxa*, *L. duciformis*, and the nothospecies *L.* ×*maoniushanensis*.

	Characters			
Taxon	Habitat	Stems	Leaf blades	Pappus
L. paradoxa	perennial herbs	proximally glabrous, distally arachnoid-puberulous	orbicular or broadly ovate, palmatisect	brown, shorter than tubular corolla and longer than its tube
L. duciformis	perennial herbs	glabrous or distally short yellow pilose	reniform or cordate, margin irregularly dentate	white, as long as tube of tubular corolla
L. ×*maoniushanensis*	perennial herbs	glabrous or sparsely pubescent above	broadly ovate, palmately parted	wine-colored, white at base, shorter than tubular corolla and longer than its tube

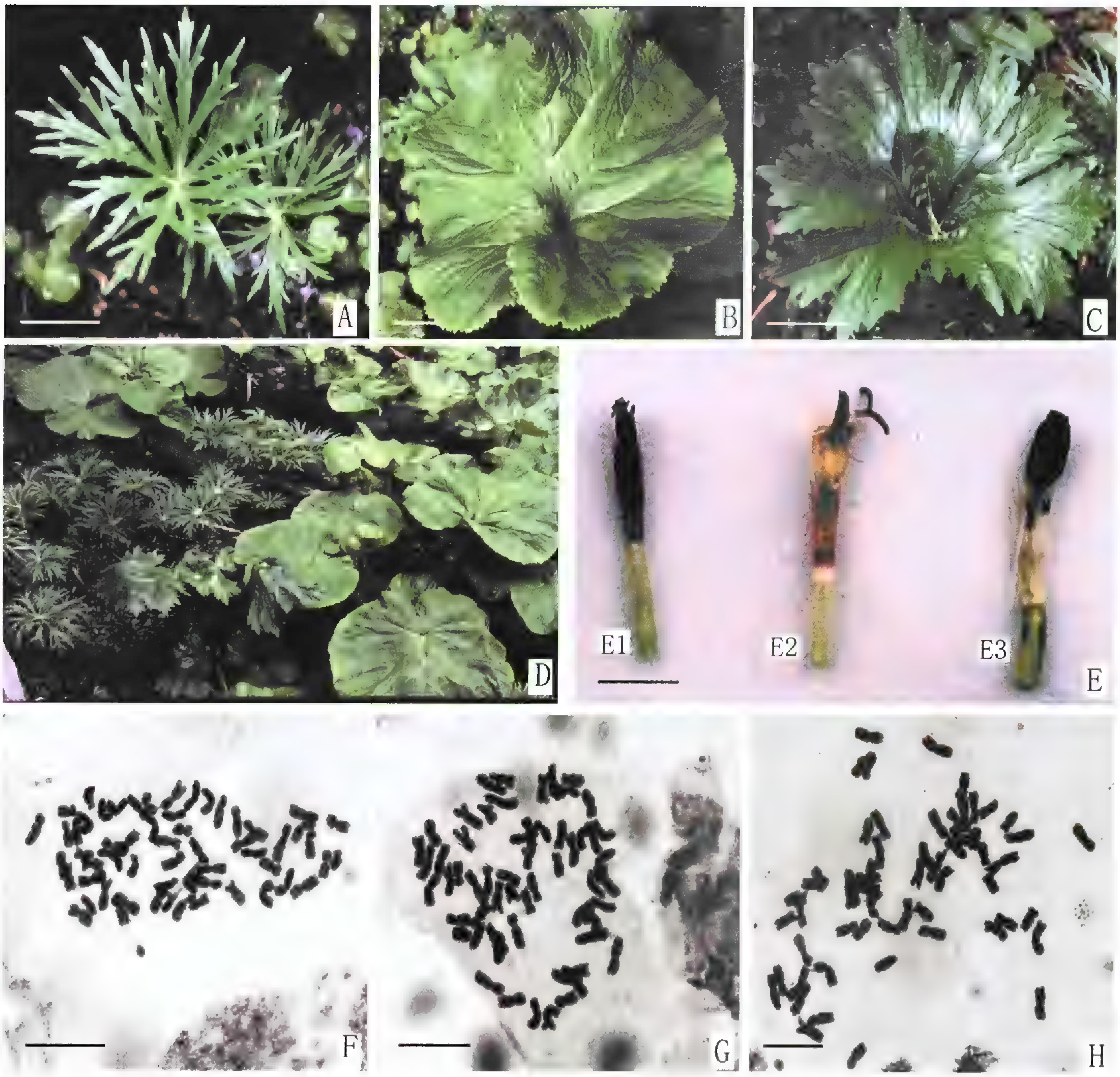

Figure 1. Photos of leaves, florets, and chromosomes of *Ligularia paradoxa*, *L. duciformis*, and *L.* ×*maoniushanensis*. —A. Leaf of *L. paradoxa*. —B. Leaf of *L. duciformis*. —C. Leaf of *L.* ×*maoniushanensis*. —D. *L. paradoxa*, *L. duciformis*, and *L.* ×*maoniushanensis* in a mixed population. —E. Florets of *L. paradoxa* (E1), *L. duciformis* (E3), and *L.* ×*maoniushanensis* (E2). —F. Somatic chromosomes of *L. paradoxa*. —G. Somatic chromosomes of *L. duciformis*. —H. Somatic chromosomes of *L.* ×*maoniushanensis*. Scale bars: A–C = 45 mm, E = 4 mm, F–H = 10 μm.

Table 2. The weight per 1000 seeds and germination rates of *Ligularia paradoxa*, *L. duciformis*, and the nothospecies *L.* ×*maoniushanensis*.

	L. paradoxa	*L.* ×*maoniushanensis*	*L. duciformis*
Weight per 1000 seeds (g)	1.17	0.79	2.26
Germination rate (%)	9.0	0	56.5

leaves of *L. paradoxa* are 3- to 8-palmatipartite (Fig. 1A, D), and those of *L. duciformis* are cordate or reniform with serrate margins (Fig. 1B, D). The pappus is white in *L. duciformis* (Fig. 1E3), and brown in *L. paradoxa* (Fig. 1E1). The morphological characters of leaf form and pappus of the putative natural hybrid are as follows: dissected peltate leaves with eight to 15 lobes (Fig. 1C, D) and wine-colored pappus (Fig. 1E2). The putative natural hybrid shares the morphological characters of its two putative parents in leaf form and pappus color.

SEED VIGOR

Embryos were observed in seeds of both *Ligularia paradoxa* and *L. duciformis*. No embryo was observed in the seeds of the putative natural hybrid. The weight and germination rates per 1000 seeds are listed in Table 2. There are distinct differences among the three taxa in the weight per 1000 seeds and in seed germination rates. No germination was observed for seeds of the putative natural hybrid, with a sample size of 1000 seeds investigated.

SOMATIC CHROMOSOME NUMBERS

The two putative parents and the putative natural hybrid are diploid and share the same chromosome number, $2x = 58$ (Table 3, Fig. 1F–H). Although the karyologies of the two putative parents are not well investigated, it is possible that hybridization takes place among taxa sharing the same chromosome number.

MEIOTIC BEHAVIOR

Chromosomes for the two putative parents displayed a normal meiotic process, characterized by the formation of 29 bivalents in diakinesis (Fig. 2A, D), with chromosome migration to two poles in anaphase I (Fig. 2B, E), and the formation of tetrads (Fig. 2C, F). However, the putative natural hybrid showed meiotic abnormalities in almost all phases, especially in diakinesis of meiotic prophase I with univalents, in anaphase I and II with lagged univalents and chromatid bridges, and in telophase II with free chromosomes (Fig. 2G–J).

ISSR MARKERS AND *TRNL-F* SEQUENCING

Although clear bands were amplified from three taxa using 10 primers, banding patterns of only four primers showed differences between the putative parents. The banding patterns of ISSR are summarized in Table 4. For example, a total of 13 bands were amplified from three taxa using the primer 807. Of these, nine bands were common to both putative parents, two bands were unique to *Ligularia paradoxa*, and two bands were unique to *L. duciformis*. The putative natural hybrid shared 12 bands with the putative parents, and no additive bands were observed (Fig. 2K).

The sequences of the chloroplast *trnL-F* region generated from the *Ligularia* hybrid and its parents were consistently 852 bp in length. No intraspecific sequence variation was observed for either parental species, although there were three nucleotide substitutions between the parental species (Table 5). All accessions of the putative hybrid had identical sequences with *L. paradoxa*. Thus, *L. paradoxa* is considered the female parent of this natural hybrid because chloroplast DNA is maternally inherited in *Ligularia* (Zhang et al., 2003).

DISCUSSION

Speciation by hybridization has been well documented in plants (Abbot, 1992; Arnold, 1992;

Table 3. Chromosome numbers and meiotic behavior in *Ligularia paradoxa*, *L. duciformis*, and the nothospecies *L.* ×*maoniushanensis* from Mt. Maoniu, Ninglang Co., Yunnan, China.

Taxon	Voucher and origin	Chromosome number	Meiotic behavior
L. paradoxa	*X. Gong 22356* (KUN)	$2n = 58$	Normal
L. duciformis	*X. Gong 22355* (KUN)	$2n = 58$	Normal
L. ×*maoniushanensis*	*X. Gong 22357* (KUN)	$2n = 58$	Abnormal

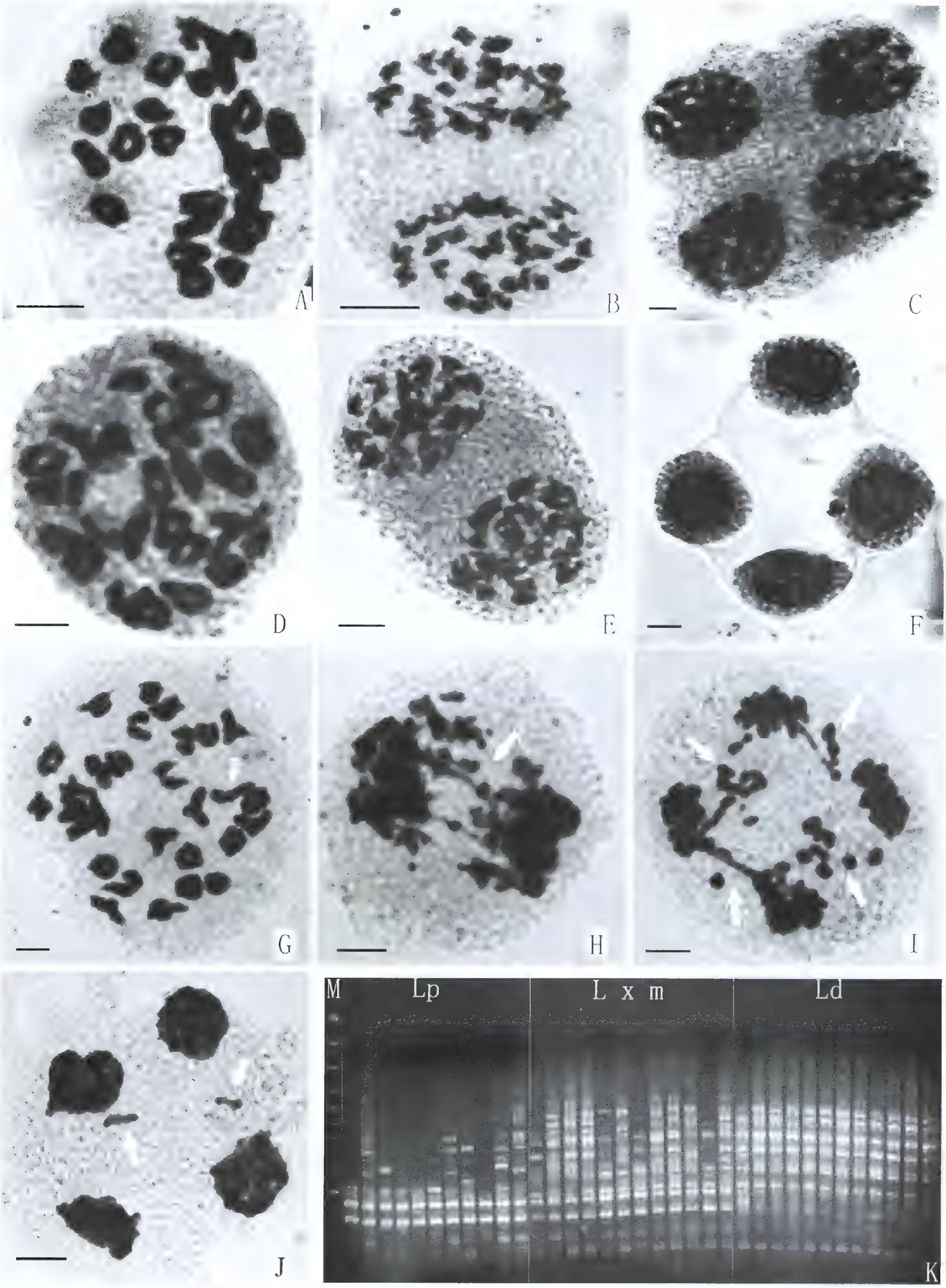

Figure 2. Meiosis of *Ligularia paradoxa*, *L. duciformis*, and the nothospecies *L.* ×*maoniushanensis*. A–C. *Ligularia paradoxa*. —A. Diakinesis. —B. Anaphase I. —C. Tetrad. D–F. *Ligularia duciformis*. —D. Diakinesis. —E. Anaphase I. —F. Tetrad. G–J. *Ligularia* ×*maoniushanensis*. —G. Diakinesis with univalents. —H. Anaphase I with lagged univalents and chromatid bridges. —I. Anaphase II with lagged univalents and chromatid bridges. —J. Tetrad with free chromosomes. —K. Banding ISSR patterns from primer 807 in *L. paradoxa* (Lp), *L. duciformis* (Ld), the nothospecies *L.* ×*maoniushanensis* (L × m), and marker banding (M). Scale bars: A–J = 10 μm.

Table 4. The banding patterns of ISSR amplified from the four primers 807(AGA GAG AGA GAG AGA GT), 823(TCT CTC TCT CTC TCT CC), 827(ACA CAC ACA CAC ACA CG), and 834(AGA GAG AGA GAG AGA G(CT)T) in *Ligularia paradoxa*, *L. duciformis*, and the nothospecies *L.* ×*maoniushanensis*. AB, number of ISSR bands additive to those for the putative natural hybrid; TB, total number of ISSR bands; UB, number of ISSR bands unique to one of the putative parents.

		ISSR primer														
		807			823			827			834			Total		
Taxon	Voucher and origin	TB	UB	AB	TB	UB	AB	TB	UB	AB	TB	UB	AB	TB	UB	AB
L. paradoxa	*X. Gong 22356* (KUN)	11	2	–	8	1	–	7	2	–	5	0	–	31	5	–
L. ×*maoniushanensis*	*X. Gong 22357* (KUN)	12	–	0	10	–	0	15	–	0	9	–	0	46	–	0
L. duciformis	*X. Gong 22355* (KUN)	11	2	–	9	2	–	13	8	–	9	4	–	42	16	–

Rieseberg, 1995). Gottlieb (1972) discussed several criteria for testing whether a particular diploid taxon originated through hybridization. These features included a geographical distribution in the region of parental sympatry, morphological intermediacy in several characters, partial fertility, biochemical additivity, and experimental synthesis of individuals resembling the hybrid. Although no single criterion can provide a clear means for testing a hypothesis of hybridization, each criterion that can be fulfilled provides a higher level of support for a hybrid origin (Gottlieb, 1972). It is also essential that hybrid criteria be evaluated carefully because morphological intermediacy and sterility are not invariably associated with hybrids (Rieseberg, 1995) and may result from entirely separate processes (Les & Philbrick, 1993). Nevertheless, Gottlieb's criteria (1972) provide a convenient avenue for discussing the putative natural hybrid between *Ligularia duciformis* and *L. paradoxa*.

In the present study, the morphological characters of leaf shape and pappus color were clearly intermediate with those of *Ligularia paradoxa* and *L. duciformis*, and the putative natural hybrid satisfies Gottlieb's (1972) criterion of morphological intermediacy. *Ligularia paradoxa*, one of the only two species with palmatipartite leaves in *Ligularia*, is restricted to Yunnan and Sichuan provinces, China, while *L. duciformis* is distributed in Yunnan, Sichuan, Hubei, Gansu, and Ningxia provinces, China (Liu, 1989; Liu et al., 1994). The two putative parents are sympatric species in geographical distribution.

The somatic chromosome numbers of the three taxa are identical ($2n = 58$). This number also agrees with reports from other species such as *Ligularia hookeri* (C. B. Clarke) Hand.-Mazz., *L. vellerea* (Franch.) Hand.-Mazz., *L. hodgsonii* Hook. and *L. kanaitzensis* (Franch.) Hand.-Mazz. (Gong et al., 2001). Therefore, the putative nothospecies is most likely a natural hybrid diploid. Both putative parents have displayed normal meiotic processes, producing seeds that germinated, suggesting that it is possible for hybridization to take place between *L. paradoxa* and *L. duciformis*.

ISSR markers are inherited in a dominant or codominant Mendelian pattern (Gupta et al., 1994; Tsumura et al., 1996) scored as diallelic, and present or absent. The absence of a band is interpreted as primer divergence or loss of a locus through the deletion of the simple sequence repeat site or chromosome rearrangement (Wolfe et al., 1998). In the present study, a total of 46 ISSR bands were amplified from three taxa using four primers. Twenty-six bands were common to both putative parents, and unique bands were distributed: five bands for *Ligularia paradoxa* and 16 bands for *L. duciformis*.

Table 5. Variable sites of the nucleotide sequences of chloroplast *trnL-F* region in *Ligularia paradoxa*, *L. duciformis*, and the nothospecies *L.* ×*maoniushanensis*.

		Variable site			
Taxon (no. of individuals)	Voucher and origin	152	214	491	GenBank accessions
L. duciformis (20)	*X. Gong 22355* (KUN)	T	T	C	DQ104429
L. paradoxa (20)	*X. Gong 22356* (KUN)	C	C	A	DQ104430
L. ×*maoniushanensis* (16)	*X. Gong 22357* (KUN)	C	C	A	DQ104431

No additional bands were observed for the putative natural hybrid, but all 46 common bands occurred in the putative natural hybrid. Therefore, ISSR markers clearly indicate that the putative natural hybrid shares a relationship with both *L. paradoxa* and *L. duciformis*.

Judging from the chromosomal behavior in meiosis and the absence of seed vigor, the putative natural hybrid may not produce sexual offspring and introgression is not likely among the three taxa. No asexual reproduction of the putative natural hybrid was noted in the field, but individuals of the putative natural hybrid at various ages were observed in the mixed population. Thus, hybridization between *Ligularia paradoxa* and *L. duciformis* has occurred more than once as observed in their zone of contact, when populations co-occur. In conclusion, the putative natural hybrid found on Mt. Maoniu is well supported as an interspecific hybrid between *L. paradoxa* and *L. duciformis*, and *L. paradoxa* is the female parent.

TAXONOMIC TREATMENT

Ligularia ×maoniushanensis X. Gong & Y. Z. Pan, nothosp. nov. ♀*Ligularia paradoxa* Hand.-Mazz. × ♂*Ligularia duciformis* (C. Winkl.) Hand.-Mazz. TYPE: China. NW Yunnan: Ninglang Co., Mt. Maoniu, 4200 m, 16 July 2001, *X. Gong 22357* (holotype, KUN!; isotype, MO not seen).

Hybrida naturalis e *Ligularia paradoxa* Hand.-Mazz. et *L. duciformis* (C. Winkl.) Hand.-Mazz. genita, ab ambabus laminis palmatifidis, magnitudine inter parentes mediis, pappis corollae vinaceis sed basi albis, seminibus abortivis facile differt.

Herbs perennial; stems simple, stout, 1.2–1.5 m tall, glabrous or sparsely pubescent above; basal and lower cauline leaves peltate, broadly ovate, 30–40 × 45–52 cm, 7- to 12-palmatipartite, 5- to 8-lobed, abaxially fulvous pubescent; 7 to 12 nerves, petioles ca. 30 cm, winged, clasping at base; upper leaves smaller. Inflorescence corymbose, peduncle 2–9 cm, densely fulvous pubescent, involucre campanulate, 10–12 × 2–3 mm, bracts in 2 series, 8 to 10 bracts, narrowly lanceolate, acute, externally densely fulvous pubescent. Ray florets absent; disc florets 5 to 7, bisexual, corolla tubular, 8–12 mm. Achenes cylindrical, glabrous; pappi wine-colored, white at base. Chromosome number $2n = 58$.

Distribution. Known only from the type specimen collected from Mt. Maoniu.

Habitat. The putative natural hybrid occurs within a mixed population of *Ligularia paradoxa* and *L. duciformis* within an area of about 2 km^2 along a stream bank in spruce-fir forest about 4200 m altitude on Mt. Maoniu in Ninglang, Yunnan, China. When we collected the specimen for the first time in July 2001, we observed 122 individuals, 116 in October 2001, and 130 in early August 2002.

IUCN Red List category. We propose that *Ligularia ×maoniushanensis* be recognized as Endangered (EN), according to IUCN Red List criteria, because of the small population size (< 250 mature individuals) in a small distribution area (< 2 km^2) (IUCN, 2001).

Phenology. Observed as flowering from July to August; fruiting from September to October.

Paratype. CHINA. **Yunnan:** Ninglang Co., Mt. Maoniu, 4200 m, 23 July 2003, *Q. T. Zhang, Y. Z. Pan & Z. Y. Yang 082306* (KUN).

Literature Cited

Abbot, R. J. 1992. Plant invasion, interspecific hybridization, and the evolution of new plant taxa. Trends Ecol. Evol. 7: 401–404.

Allen, G. A. 2002. Hybrid speciation in *Erythronium* (Liliaceae), a new allotetraploid species from Washington State. Syst. Bot. 26(2): 263–272.

Aparicio, A., R. G. Albaladejo, M. Porras & G. Cebollas. 2000. Isozyme evidence for natural hybrid in *Phlomis* (Lamiaceae): Hybrid origin of the rare *P. ×margaritae*. Ann. Bot. (Oxford) 85: 7–12.

Arnold, M. L. 1992. Natural hybridization as an evolutionary process. Annual Rev. Ecol. Syst. 23: 237–261.

———. 1997. Natural Hybridization and Evolution. Oxford University Press, New York.

Barton, N. H. & G. M. Hewitt. 1989. Adaption, speciation and hybrid zones. Nature 341: 497–503.

——— & ———. 2001. The role of hybridization in evolution. Mol. Ecol. 10: 551–568.

Brochmann, C., T. Nilsson & M. T. Gabrielsen. 1996. A classic example of postglacial allopolyploid speciation re-examined using RAPD markers and nucleotide sequences: *Saxifraga osloensis* (Saxifragaceae). Symb. Bot. Upsal. 31: 75–89.

Choi, K., J. S. Kim & J. H. Pak. 2001. Natural hybridization between *Pseudostellaria davidii* and *Pseudostellaria palibiniana* (Caryophyllaceae). Pl. Spec. Biol. 16: 39–47.

Doyle, J. J. & J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19: 11–15.

Ellstrand, N. C., R. Whitkus & L. H. Rieseberg. 1996. Distribution of spontaneous plant hybrids. Proc. Natl. Acad. Sci. U.S.A. 93: 5090–5093.

Gong, X., Z. J. Gu, X. Y. Lu & C. Q. Zhang. 2001. The karyotypes of seven species in *Ligularia*. Acta Bot. Yunnan. 23(2): 216–222.

Gottlieb, L. D. 1972. Levels of confidence in the analysis of hybridization in plants. Ann. Missouri Bot. Gard. 59: 435–446.

Grant, V. 1981. Hybrid Speciation. Columbia University Press, New York.

Gupta, M., Y. S. Chyi, J. Romero-Severson & J. L. Owen. 1994. Amplification of DNA markers from evolutionary diverse genomes using simple-sequence repeats. Theor. Appl. Genet. 89: 998–1006.

Handel-Mazzetti, H. 1938. Die Chinesischen Arten der Gattung *Ligularia*. Bot. Jahrb. Syst. 69: 95–142.

Harris, S. A. & R. Ingram. 1992. Molecular systematics of the genus *Senecio* L: I. Hybridization in a British polyploid complex. Heredity 69: 1–10.

Hu, S. Y. 1967. The Compositae of China. Quart. J. Taiwan Mus. 20: 53–77.

IUCN. 2001. IUCN Red List Categories and Criteria Version 3.1. Prepared by the IUCN Species Survival Commission. IUCN, Gland, Switzerland, and Cambridge, United Kingdom.

Les, D. H. & C. T. Philbrick. 1993. Studies of hybridization and chromosome number variation in aquatic angiosperms: Evolutionary implications. Aquatic Bot. 44: 181 228.

Levin, D. A. 1983. Polyploidy and novelty in flowering plants. Amer. Naturalist 122: 1–25.

Liu, S. W. 1985. The taxonomic system of the genus *Ligularia* (L.) Cass. Bull. Bot. Res., Harbin. 5(4): 63–80.

———. 1989. Flora Reipublicae Popularis Sinicae, Vol. 79(1). Science Press, Beijing.

———, D. S. Deng & J. Q. Liu. 1994. Origin, evolution, and geography of *Ligularia* (L.) Cass. Acta Phytotaxon. Sin. 32(6): 514–524.

Padgett, D. J., D. H. Les & G. E. Crow. 1998. Evidence for the hybrid origin of *Nuphar* ×*rubrodisca* (Nymphaeaceae). Amer. J. Bot. 85: 1468–1476.

Rieseberg, L. H. 1995. The role of hybridization in evolution: Old wine in new skins. Amer. J. Bot. 82: 944–953.

———. 1997. Hybrid origins of plant species. Annual Rev. Ecol. Syst. 28: 359–389.

Sang, T., D. J. Crawford & T. F. Stuessy. 1997. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). Amer. J. Bot. 84: 1120–1136.

Soltis, P. S., D. E. Soltis & P. G. Wolf. 1991. Allozymic chloroplast DNA analyses of polyploidy in *Polystichum* (Dryopteridaceae): I. The origins of *Polystichum californicume* and *Polystichum scopulnum*. Syst. Bot. 16: 245–256.

Soltis, D. E. & P. S. Soltis. 1993. Molecular data and the dynamic nature of polyploidy. Crit. Rev. Pl. Sci. 12: 243–273.

Stebbins, G. L., Jr. 1959. The role of hybridization in evolution. Proc. Amer. Philos. Soc. 103: 231–251.

———. 1971. Chromosome Evolution in Higher Plants. Edward Arnold Publishers Ltd., London.

Sun, M. 1996. The allopolyploid origin of *Spiranthes hongkongensis* (Orchidaceae). Amer. J. Bot. 83: 252–260.

Taberlet, P., L. Gielly, G. Pauton & J. Bouvet. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Pl. Mol. Biol. 17: 1105–1109.

Tsumura, Y., K. Ohba & S. H. Strauss. 1996. Diversity and inheritance of inter-simple sequence repeat polymorphisms in Douglas fir (*Pseudotsuga menziesii*) and sugi (*Cryptomeria japonica*). Theor. Appl. Genet. 92: 40–45.

Wendel, J. F. 1989. New world tetraploid cottons contain old world cytoplasm. Proc. Natl. Acad. Sci. U.S.A. 86: 4132–4136.

Wolfe, A. D., Q. Y. Xiang & S. R. Kephart. 1998. Assessing hybridization in natural populations of *Penstemon* (Scrophulariaceae) using hypervariable intersimple sequence repeat (ISSR) bands. Mol. Ecol. 7: 1107–1125.

Zhang, Q., Y. Liu & Sodmergen. 2003. Examination of the cytoplasmic DNA in male reproductive cells to determine the potential for cytoplasmic inheritance in 295 angiosperm species. Pl. Cell Physiol. 44: 941–951.